

# Somatic Cell Gene Mutations in Humans: Biomarkers for Genotoxicity

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Somatic cell gene mutations arising *in vivo* in humans provide biomarkers for genotoxicity. Four assays, each measuring changes in a different "recorder" gene, are available for detecting mutations of the hemoglobin (*Hb*) and glycophorin A (*gpa*) genes in red blood cells and the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) and HLA genes in T-lymphocytes. Mean adult background mutant frequencies have been established; i.e., approximately  $4 \times 10^{-8}$  (*Hb*),  $5-10 \times 10^{-6}$  (*hprt*),  $10-20 \times 10^{-6}$  (*gpa*) and  $30 \times 10^{-6}$  (HLA). All the assays have now been used in studies of individuals exposed to physical and/or chemical genotoxic agents, and all have shown elevated values following exposures; examples are presented. In addition to quantitation, the lymphocyte assays allow molecular analyses of *in vivo* mutations, the definition of background and induced mutational spectra, and the search for unique changes for characterizing specific mutagens. The *HPRT* system currently has the largest database in this regard. Approximately 15% of adult background *hprt* mutations are due to gross structural alterations (primarily deletions) having random breakpoints; 85% result from "point" changes detected only by sequencing. In contrast, a specific intragenic deletion due to DNA cleavage at specific sites characterizes fetal *hprt* mutations, implicating a developmental mistake in their genesis. (This kind of developmental mistake in other genes is frequently observed in lymphoid malignancies.) Mutational spectra are just beginning to be defined for induced *hprt* mutations, e.g., ionizing radiation produces large deletions. Characterization of HLA mutations at the molecular level, accomplished thus far at the level of Southern blots, shows that approximately 30% result from events that render the gene "homozygous," i.e., somatic recombination. Details of the molecular aspects of *in vivo* gene mutations in human lymphocytes are described.

## Introduction

Environmental mutagens can produce disease in individuals by "inducing" lesions in critical genes of somatic cells. If the genes are important in malignant transformation, the disease is cancer. Other diseases may also have an environmental basis. Measures of induced or spontaneous genetic damage arising *in vivo* in human somatic cells have public health relevance as early warning detectors for such exposures.

Environmentally induced lesions in cells may be manifest at any level of organization of the genetic material, i.e., the chromosome, the gene, or the primary DNA level. Assays are available for quantifying and characterizing all such damage. At the chromosome level, technical advances in recent years have increased the rapidity and precision of measures of aberrations and micronuclei. For primary DNA lesions, there is a variety of methods for measuring

chemical adducts, other lesions, or cellular repair responses that reflect such lesions (1).

Genetic lesions in somatic cells are processed, resulting in misinformation (or no information) encoded by the involved region of DNA. Broadly speaking, such processed lesions are called "mutations." Chromosome-level genetic damage may result in a mutation if it is not lethal to the cell and disrupts or deletes a gene or its controlling elements. Similarly, primary DNA lesions, while not themselves mutations, may be processed to produce changes in somatic genetic coding sequences. The final common pathway for somatic genetic damage to be of functional (i.e., pathogenic) significance is a heritable alteration in genetic information, i.e., a somatic gene mutation. Measures of somatic mutations are relevant for human population monitoring because they measure this common functional pathway. When these mutations occur in critical regions, they produce genotoxic disease.

Most studies of human genotoxic diseases are concerned with genes involved in the disorder. Cancer research has become highly focused on the critical genes involved in malignant transformation. Certainly, studies of specific oncogenes and tumor-suppressor genes are indis-

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pensable for an understanding of pathogenesis, i.e., for relating gene malfunctions to disease states. However, genetic lesions arising in cancer-associated genes are rare, usually nonselectable, and, when they do arise, may alter cell proliferation characteristics such that their primary frequencies become difficult to measure. For investigations of the mutation process itself and for monitoring human exposures to environmental mutagens, recourse is made to somatic mutations in simple "housekeeping" or other genes that result in easily recognized cellular phenotypic changes. Such somatic mutations serve as recorders. Of course, ultimately, correlations must be made between mutations in recorder genes and genotoxic diseases in monitored populations for the former to have validity for human risk assessment.

This review briefly considers and compares all currently available assays for measuring *in vivo* somatic cell gene mutations in humans. Special emphasis is given to hypoxanthine-guanine phosphoribosyltransferase (*hprt*) mutations in T-lymphocytes for three reasons: it is used by several laboratories, it has the largest current database, and information is available regarding molecular characterizations of the mutations arising *in vivo*.

## Assays for Somatic Cell Mutations Arising *in Vivo* in Humans

There currently are four assays for detecting *in vivo* somatic cell gene mutations in humans. All measure altered cellular phenotypes. For obvious reasons, the current assays study mutations in blood cells; two in red blood cells (RBCs) and two in T-lymphocytes.

### Somatic Mutations in Red Blood Cells

Somatic mutations of the hemoglobin (*Hb*) genes and the glycophorin-A (*gpa*) gene are reflected as altered phenotypes in RBCs. Although the concept of monitoring *in vivo* somatic mutations by studying changes in Hb came first, an automated assay for actually scoring mutants was only recently developed (2,3). By far, most studies of *in vivo* mutations detected as RBC changes have used the *GPA* assay.

Since mature RBCs in mammals do not have a nucleus, the mutations that produce phenotypic alterations must arise at an earlier stage of differentiation, i.e., in nucleated bone marrow progenitor cells. Genotoxicants must reach this body compartment to exert their effects. Bone marrow derived mutants are then amplified and recognized as RBCs in peripheral blood. This relationship between mutational events and mutant frequencies must be understood to interpret mutagenicity studies using this assay. At least some of the mutations induced in bone marrow precursor cells will arise in multipotent stem cells that produce cells that begin the differentiation process and cells that renew the stem-cell compartment. Because of the latter, mutations occurring in multipotent cells are reflected as mutants throughout the life of the individual, i.e., have "long memory." (Those arising in more differentiated cells will eventually die out.) This long memory may or

may not be an advantage, depending on whether monitoring is for genotoxic exposures in the recent or distant past.

A further consequence of RBCs not having a nucleus is that there is no possibility for molecular studies. Therefore, indirect methods must be used to verify the mutational basis of the phenotypic changes and molecular mutational spectra cannot be determined. RBCs with altered phenotypes are often referred to as "variants."

At present, mutation of *Hb* genes is determined only as the specific mutations of the wild-type *Hb $\beta$*  gene (*HbA*) to three mutant Hb forms, i.e., HbS, Hb San Jose, and Hb Leiden. The *Hb $\beta$*  gene is on chromosome 11p and spans 2 kb (4,5). However, the specific mutations are much more restricted changes, i.e., either specific base changes, frameshifts, or small deletions. This gives a relatively small target for mutation, as reflected in the low background variant frequencies in normal adults, i.e.,  $5-10 \times 10^{-8}$ . Rare RBCs containing mutant Hb in the heterozygous state in HbA/HbA individuals are detected by automated image analysis of cells fixed on slides and treated with fluorescent antimutant Hb polyclonal antibodies (6).

Glycophorin A is the cell surface protein on RBCs that defines the M and N blood group antigens. The M and N groups differ by two nonadjacent amino acids, are codominantly expressed on RBCs, and are specified by the only two alleles at the *gpa* locus (7). The *gpa* mutants are recognized on rare RBCs of an M/N constitutional heterozygote as either the simple loss of M or N expression or loss of expression of one (M or N), with double expression of the other. Although the system has the disadvantage that only M/N heterozygous individuals are informative, the M and N gene frequencies are approximately equal in all populations so that heterozygotes are approximately 50% of all individuals. The simple loss of M or N expression (called "hemizygous" mutation) is thought to arise from allele inactivation somatic mutation (e.g., point mutation or deletion), whereas loss of one with double expression of the other (called "homozygous" mutation—not be confused with constitutional homozygote) is believed to represent somatic recombination, gene conversion, or chromosome loss with duplication. The *gpa* locus is on chromosome 4q, spans 44 kb and has 4 exons (8). It is a large target for mutation, as reflected in background mutant frequencies in normal adults, which are of the order of  $10-20 \times 10^{-6}$  for both hemizygous and homozygous mutants. These *gpa* mutants (variants) are enumerated by the use of a flow cytometer with RBCs that have been treated with highly specific anti-M and anti-N antibodies linked to green and red fluorophors, respectively (9,10).

The RBC assays have several advantages for human population monitoring. Because RBCs are so abundant in blood, relatively small samples suffice for analysis. The Hb assay measures precise gene alterations, making it virtually impossible for nongenetic phenotypic changes ("phenocopies") to produce the variant cells scored as mutants. However, the precise nature of these requisite mutations is a difficulty, i.e., mutant frequencies are so low that large numbers of cells must be scored to achieve statistical reliability, and some types of genetic change will

not be detected. The former problem may be partially overcome by mixing several antimutant Hb antibodies when treating fixed cells, thereby measuring several different *Hb $\beta$*  mutations simultaneously. The use of an image analyzer greatly enhances the utility of this assay. The GPA assay has several advantages for human monitoring. It is extremely rapid, allowing for large numbers of individuals to be easily studied. Also, it allows for recognition of potentially important genetic lesions such as somatic recombination because of the autosomal location and large target characteristics of the gene.

## Somatic Mutations in T-Lymphocytes

Somatic mutations arising *in vivo* in the *hprt* gene or in one or more of the HLA genes can be recognized in human T-lymphocytes. A short-term assay for quantitating the former was described almost 15 years ago, and a cloning assay for *hprt* mutations became available almost 10 years ago (11-13). Several groups now use one or the other of these assays for human studies (14,15). A cloning assay for studying HLA mutations arising *in vivo* in T-lymphocytes was described almost 5 years ago (16).

One advantage of T-lymphocytes for *in vivo* mutagenicity studies is that, when the cloning assay is used, *in vivo*-derived mutants can be isolated, propagated *in vitro*, and the mutation characterized at the molecular level. A large database containing such information is developing for *hprt* mutations where the background molecular mutational spectrum in nonmutagenic individuals is being characterized for comparison with spectra observed after various environmental exposures (17). The hope is that mutagens or classes of mutagens will produce sufficiently characteristic spectra of *hprt* damage to serve as signatures for that specific exposure. If this hope is realized, qualitative studies of *in vivo* somatic mutations may be more informative than quantitative studies.

The T-lymphocyte population *in vivo* has enormous heterogeneity. At the extreme, this population consists of millions of individual *in vivo* clones, each identified by the specific T-cell receptor (TcR) used by all members of that clone. Specific T-cell receptors are dimeric cell-surface proteins through which individual T-cells recognize foreign antigen. Specific T-cell receptors are specified by *TcR* genes that themselves undergo somatic diversification by a process of rearrangement of segments of germ-line genes. The TcR rearrangement pattern of a given clone, fixed during thymic differentiation, persists throughout the life of the individual. Each of the millions of individual TcR-defined T-cell clones is thereby characterized by a specific and identifiable rearrangement of *TcR* genes. This can be used to advantage in that molecular analyses of *TcR* genes in T-cell mutants (selected on the basis of their mutation of some other gene, e.g., *hprt* or HLA) defines the randomness of the somatic mutation process or, conversely, its clonality, i.e., tendency to occur in a nonrandom manner among clones. Thus, the clonal distribution of somatic mutation can be established (18).

As for RBCs, the *in vivo* kinetics of T-cells make interpreting mutation studies complex. T-lymphocytes arise

in the bone marrow, undergo maturation (rearrangement of *TcR* genes) in the thymus, and then have a prolonged life span in peripheral tissue, e.g., in lymph nodes, spleen, gut. They travel from site to site via the blood. This traffic from bone marrow to thymus to periphery is pronounced during fetal life and early childhood, becomes less pronounced with age, and may terminate in late adolescence. There are few or no stem cells as such in the adult; the long-lived T-cells in the periphery maintain the T-lymphocyte population, and there is no further addition to clonal diversity. Most T-cells in the periphery at any given time are quiescent, i.e., in the G<sub>0</sub> phase of the cell cycle, but are intermittently stimulated to undergo cell division and clonal amplification, usually by antigen.

Somatic mutations in T-lymphocytes may occur at several differentiation stages in the fetus and children, but probably can arise only in the periphery (i.e., in mature T-cells) in adults. Because peripheral T-cells are present in virtually all body compartments, mutagens can exert their effects on these cells without having to reach the bone marrow compartment. Mutations arising in the periphery, however, do not have long memory, and T-cell mutations in adults will probably produce transient increases in *in vivo* mutant frequencies. This is advantageous or disadvantageous, depending on the purpose of the study.

The *hprt* gene is on the X-chromosome (19). Thus, it is hemizygous, either actually (males) or functionally (females). The *hprt* gene is a constitutive but dispensable housekeeping gene that codes for an enzyme (HPRT) that phosphoribosylates hypoxanthine and guanine for purine salvage. HPRT activity is also able to phosphoribosylate purine analogues, e.g., 6-thioguanine (TG), to render them cytotoxic. Inactivating mutations of the *hprt* gene allow the mutant cells to grow in the presence of otherwise toxic concentrations of purine analogues, providing a convenient means for selection. The X-chromosomal location of the *hprt* gene is advantageous in obviating dominance-recessive considerations, but disadvantageous in that important genotoxic events such as somatic recombination cannot be detected. The *hprt* gene spans 44 kb and includes nine exons, making it a large target for mutation as reflected in background adult mutant frequency values that average  $5-10 \times 10^{-6}$  (20).

Two classes of assays are available for quantitating *in vivo* *hprt* mutations. One involves a short-term assay using autoradiography or immunofluorescence to detect <sup>3</sup>H [thymidine] or bromodeoxyuridine (BrdU) incorporation, respectively, in mutant (variant) cells that are resistant to TG inhibition of first-round phytohemagglutinin (PHA)-stimulated DNA synthesis *in vitro* (11,21). These short term assays are simple, relatively inexpensive, and have the potential for automation.

The second assay for *hprt* mutant T-cells uses direct cloning. T-cells are cultured in limiting dilutions in the presence and absence of TG selection (12,13). The ratio of cloning efficiency with TG to cloning efficiency without TG defines the *hprt* mutant frequency. As noted, mutant colonies can be isolated and propagated *in vitro* for further analysis. The HPRT cloning assay quantitative results are quite similar to those obtained with the short-term assays.

The HLA gene complex includes several linked loci containing two classes of genes that encode tissue antigens, which are cell-surface recognition molecules of importance in immune responses. There are many alleles at each of the HLA loci, resulting in marked population polymorphism. Mutational loss of an antigen specified by one allele at each of these loci can be easily detected in constitutionally heterozygous individuals. The HLA gene complex is on chromosome 6p. Its autosomal location allows it to recognize potentially important genotoxic events such as gene conversion and somatic recombination. Thus far, only mutations in two alleles of one class of HLA genes have been studied, i.e., HLA-A2 and HLA-A3. This HLA gene spans 5 kb and contains 7 exons, making it another large target for mutation. *In vivo* mutant frequencies average approximately  $20\text{--}30 \times 10^{-6}$  for normal adults (16).

An assay for *in vivo*-derived HLA loss mutants involves direct cloning, as described for *hprt* mutations. However, rather than selecting with TG, T-lymphocytes are first treated with the appropriate anti-HLA antibody and complement to kill nonmutant cells. Care must be taken that immunoselection is complete. Calculations for mutant frequencies are analogous to those for determining *hprt* mutant frequencies. HLA mutant T-cells can be isolated, propagated, and characterized.

## Quantitative Assessment of *in Vivo* Somatic Mutations in Humans

There have now been sufficient quantitative studies of *in vivo* somatic mutations in humans using one or more of the assays described to permit a comparison of results.

### Background (Spontaneous) Variant/Mutant Frequencies

Several studies have defined background (spontaneous) variant/mutant frequencies in nonexposed normal individuals, the effects of age and smoking in these individuals, and variant/mutant frequency values in individuals with cancer-prone conditions thought to be due to DNA repair deficiencies. A summary of these quantitative results is given in Table 1. Background variant/mutant frequency values using the several assays reflect, in general, the size of the genetic target and, perhaps, the kinds of mutational events that are scored by that assay. Therefore, background variant frequencies at the *Hbb* locus reflect specific mutations in codons, i.e., a single transversion for HbS, a single transition for Hb San Jose, or a specific small deletion for Hb Leiden. The mean Hb variant frequency for normal adults is  $1\text{--}5 \times 10^{-8}$ /specific mutation (6). Background variant frequencies at the *gpa* locus reflect a

Table 1. Comparative results of variant/mutant frequencies for the different *in vivo* somatic cell gene mutation assays in adult humans (unexposed).

	RBC assays		T-lymphocyte assays		
	Hb	<i>gpa</i>	<i>hprt</i> Short term	Cloning	HLA
<b>Normals</b>					
Mean background values	$1\text{--}5 \times 10^{-8a}$ (6)	$10 \times 10^{-6}$ hemizygous $10 \times 10^{-6}$ homozygous (22)	$5\text{--}10 \times 10^{-6}$ (21,23-25)	$5\text{--}10 \times 10^{-6}$ (17)	$30 \times 10^{-6}$ (16,26)
Age effect	NT	↑ 2%/year (9,27)	↑ 5%/year (25)	↑ 1.6-5%/year (28-31)	↑ (26)
Smoking effect*	— (31)	↑ 30% (32)	↑ (25,33)	↑ 56% (28,31)	NT
<b>DNA repair defects (constitutional homozygotes)</b>					
Ataxia telangiectasia	— (31)	↑ <sup>b</sup> (34)	NT	↑ (35)	NT
Xeroderma pigmentosum	— (31)	— (22)	NT	↑ (36)	NT
Bloom syndrome	NT	↑ (37)	↑ (38)	NT	NT
Fanconi anemia	NT	NT	↑ (39)	↑ (39)	NT
Werner syndrome	NT	NT	↑ (40)	NT	NT

Abbreviations: RBC, red blood cells; Hb, hemoglobin; GPA, glycophorin-A; HPRT, hypoxanthine-guanine phosphoribosyltransferase; ↑, variant/mutant frequency increase; NT, not tested; —, no change.

<sup>a</sup>Rate per codon in *Hbb* gene: transversion for HbS, transition for Hb San Jose, and deletion for Hb Leiden.

\*Not statistically significant in all studies.

<sup>b</sup>Increase was primarily in hemizygous mutants.

variety of inactivating mutations giving hemizygous variants and events akin to somatic mutation giving homozygous variants (22). The background mean frequency of each of these is approximately  $10 \times 10^{-6}$ , i.e., more than two orders of magnitude greater than for Hb variants. Mean *hprt* variant (short-term assay) and *hprt* mutant (cloning assay) frequencies have been established for normal nonmutagen-exposed adults. These are approximately the same, i.e.,  $5-10 \times 10^{-6}$ , as might be expected since they each measure *in vivo* mutation of the same gene, which is large target (17,21,23-25). Some genetic events however (e.g., somatic recombination), are not detectable at *hprt*. Finally, the mean background mutant frequency at HLA is approximately  $30 \times 10^{-6}$ , which reflects the large target size and the ability to record somatic exchanges (16,26). The relative order of mean background variant/mutant frequencies for normal adults is  $Hb < hprt < gpa < HLA$ .

Superimposed on these mean background frequencies may be age effects and smoking effects. Although, strictly speaking, smoking is a mutagen exposure, it is so ubiquitous that it must be accounted for in human population monitoring. An increase in variant/mutant frequency with age has been found for all assays tested (9,25-31). Smoking has been found to increase mean *gpa* variant and *hprt* variant and mutant frequency values (25,28,31-33). The magnitude of increase, however, varied from study to study and did not always reach statistical significance. There is no report of this having been tested for HLA mutations, and there was no smoking related increase in a small study of *Hb* somatic mutations.

Studies of *in vivo* somatic mutations in individuals with DNA deficiencies have been particularly interesting. Both *gpa* variant and *hprt* mutant frequencies are increased in patients with ataxia telangiectasia [AT (34,35)]. The increase for *gpa* is primarily for hemizygous variants. There was no increase in Hb variant frequencies in AT patients (31). For xeroderma pigmentosum (XP), there was no increase in variant frequency for either *Hb* or *gpa*, but a clear increase in mutant frequency for *hprt* (22,31,36). It is tempting to think that this reflects the necessity of the mutagen reaching the bone marrow compartments for the production of RBC precursor mutations, and the ability of lymphocytes to circulate through skin and be subject to UV effects. HLA mutant frequencies have not been determined in XP patients. For Bloom syndrome, both *gpa* and *hprt* variant frequencies were increased, with no mutagenicity studies reported for the other assays (37,38). Unlike in AT, however, both hemizygous and homozygous *gpa* variant frequencies were increased in Bloom syndrome, perhaps reflecting the marked chromosome level changes seen in the disorder. A single study has reported increased *hprt* variant and mutant frequencies in some patients with Fanconi anemia and another an increase in *hprt* variant frequency in individuals with Werner syndrome (39,40). In all of the DNA repair deficiency conditions tested, the constitutional heterozygotes had, on average, normal background variant/mutant frequency values.

## Induced Variant/Mutant Frequencies

Several studies have investigated the effects of exposures to genotoxic agents in adult humans. These include ionizing irradiations (Table 2) and several chemical agents (Table 3).

Exposure to external beam ionizing irradiation results in increases in *hprt* mutant frequencies in both radiotherapy technicians and patients, but not in increases in *gpa* variant frequencies (32,33,41-44). Atomic bomb survivors studied 45 years after exposure showed increases in both *hprt* mutant and *gpa* variant frequencies (15,45-47). Both showed a dose-response relationship with exposure level and the slope was higher for the *gpa* response. This latter difference may reflect the different sites of mutations in RBCs and T-lymphocytes, and the potential long life of RBC stem-cell mutants. However, the increased frequency of *hprt* mutants also demonstrates the long life of some mutant T-cells *in vivo*. An increase in the frequency of Hb, *gpa*, and *hprt* variants has been reported in individuals exposed to radiation as a result of accidental environmental contamination (6,24,48-50).

Internal exposure to technetium-99m through radionuclide angiography has yielded conflicting results in studies of *hprt* mutant frequency (51,52). However, internal exposure to yttrium-90 or iodine-131 through a radioimmunotherapy (RIT) procedure clearly resulted in increases in the *hprt* mutant frequency, which correlated with the initial doses of administered radioactivity (53,54). (A specific spectrum of mutations was also found, as described below.) Lastly, household exposure to radon has been shown to yield a correlation between increased *hprt* mutant frequency and total exposure (55).

Exposure to chemical agents such as those in combination chemotherapy results in an increased frequency of *gpa* variants and *hprt* mutants (56-58). An increase in *hprt* variant frequency was found in two studies of the effects of exposure to cyclophosphamide, either therapeutic or occupational (23,59). Occupational exposure to ethylene oxide resulted in increases in both the Hb variant and *hprt* mutant frequency (60,61).

When the studies were able to access the longitudinal stability of the increased variant/mutant frequencies, elevations were generally found to be transient over time. Thus, the assays appear to be able to detect recent exposures to genotoxic agents. However, the effects of exposures in the more distant past to ionizing irradiation are also clearly demonstrated. Chronic exposures, as in the radon study, may be most relevant for human risk assessment, and more studies of this type are needed.

## Molecular Analyses of *in Vivo* Somatic Mutations in Humans

*Hprt* and HLA mutations arising *in vivo* in T-lymphocytes may be analyzed at the molecular level using techniques such as direct Southern blotting or genomic polymerase chain reaction (PCR) amplification to define major structural alterations and the direct PCR of cDNA and sequencing to characterize point mutations (62-64). In

Table 2. Comparative results of variant/mutant frequencies for the different *in vivo* somatic cell gene mutation assays in adult humans (ionizing radiation effects).

Group	RBC assays		T-lymphocyte assays	
	Hb	<i>gpa</i>	<i>hprt</i>	
			Short term	Cloning
Radiation technicians	NT	NT	NT	↑ (41,42)
Radiation therapy	NT	— (32)	↑ (33)	↑ (43,44,53,54)
Atomic bomb	NT	↑ (45,46)	NT	— (57)
Radiation accidents	↑ (6)	↑ (48,49)	↑ (24,50)	↑ (15,47)
Radionuclide	NT	NT	NT	NT
angiography				↑ (51)
Radon (household)	NT	NT	NT	— (52)
				↑ (55)

Abbreviations: RBC, red blood cells; Hb, hemoglobin; GPA, glycophorin-A; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NT, not tested;

↑, variant/mutant frequency increase; —, no change.

<sup>a</sup>HLA assay not conducted for any group.

Table 3. Comparative results of variant/mutant frequencies for the different *in vivo* somatic cell gene mutation assays in adult humans (chemical effects).

	RBC assays		T-lymphocyte assays	
	Hb	<i>gpa</i>	<i>hprt</i>	
			Short term	Cloning
Combination chemotherapy	NT	↑ (56)	NT	↑ (57,58)
Cyclophosphamide	NT	NT	↑ (23,59)	— (44)
Ethylene oxide	↑ (60)	NT	NT	NT
				↑ (61)

Abbreviations: RBC, red blood cells; Hb, hemoglobin; GPA, glycophorin-A; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NT, not tested;

↑, variant/mutant frequency increase; —, no change.

<sup>a</sup>HLA assay not conducted for any group.

addition, studies of *TcR* gene rearrangement patterns among mutants from a given individual establish clonal distributions of *in vivo*-derived mutants (65).

## Background Mutations

Approximately 15% of the background *in vivo hprt* mutations in adults show gross structural alterations visible on Southern blots. Most of these alterations are deletions. Only the minority, however, are total (i.e., involving all nine exons of *hprt*). Breakpoints are apparently randomly distributed throughout the gene with no hot-spots thus far identified.

The remaining 85% of background *in vivo hprt* mutations in adults are due to point mutations, i.e., base changes, frameshifts, splice-site alterations, or small deletions or insertions, involving less than the 500 bp necessary for detection on Southern blots. A repository is being maintained by Thomas Skopek at the University of North Carolina at Chapel Hill to collate all data on such *hprt* point mutations. This repository now includes information concerning both spontaneous (background) and induced *in vivo* and *in vitro* mutations in human cells. Overall, the most common point mutations observed at *hprt* are base substitutions (85% missense, 13% nonsense, 2% no amino acid change), followed by splice site mutations, frameshifts, and small deletions. Every class of base substitution has been observed at similar frequencies, and these have been distributed throughout all nine exons. Splice site mutations have involved all exons except exon 1. Frame-

shift mutations, in general, occur at or adjacent to sequences containing two or more consecutive identical bases, whereas small deletions, involving from 2 to 49 bp, are often flanked by 2–5 base direct repeat sequences.

The background *in vivo hprt* T-cell point mutations have thus far not shown a characteristic pattern or spectrum. However, several hot-spots have been observed, i.e., sites at which mutation has occurred more than once. Given the large target size of *hprt*, the current inability to define a spontaneous mutational spectrum is not unexpected; the total number of background *in vivo hprt* mutations analyzed to date has simply not been sufficient.

Unexpectedly, the background *hprt* mutational spectrum of newborn humans, determined from T-cell mutants isolated from placental blood and reflecting mutations in the fetus, is quite different from that determined for adults. Rather than 15% showing gross structural alterations on Southern blots, up to 85% of fetal mutations show such gross changes (66,67). Furthermore, while adult breakpoints for gross alterations are randomly distributed within the *hprt* gene, fetal mutations have a characteristic partial deletion that involves only exons 2–3. These mutations have been shown to contain specific breakpoints in introns 1 and 3, which is consistent with the known specificity of the VDJ recombinase activity (68). Molecular analyses of *in vivo* HLA T-cell mutations are also in progress. Southern blot studies of background mutants from nonexposed adults reveal that approximately 30% result from total gene deletions. Significantly, the use of chromosome 6p polymorphic DNA probes further shows

another 30% to be homozygous for markers for which the individual is constitutionally heterozygous. The most likely interpretation is that these *in vivo* background HLA mutations result from a process akin to somatic recombination (69,70). If so, HLA becomes an important recorder for such events. At present, molecular studies of *in vivo* HLA mutations are being pursued by a single group. There are no published molecular characterizations of *in vivo* fetal mutations, or of the remaining 30+ % of *in vivo* background mutations in adults that appear to be point mutations.

## Induced Mutations

The only molecular mutational spectrum thus far defined for induced *in vivo* somatic mutations in humans is for *hprt* in individuals following exposures to ionizing radiation (53,54). The exposed individuals are cancer patients receiving radioimmunoglobulin therapy (RIT). RIT involves the intravenous administration of a radionuclide linked to an antibody that localizes to tumor and other body tissues, thus providing chronic total body irradiation in an exponentially decaying dose. Two hundred forty-one independent *in vivo hprt* mutational events occurring in 19 RIT patients, 118 independent *in vivo hprt* mutations in four cancer patients before RIT, and 287 background mutations in normal individuals were analyzed by Southern blots and the results compared. The mean percent of gross structural alteration mutations in the radiation-exposed group was 38.3%, compared to 16.9% in cancer patients before RIT and 14.2% in normal adults. Thus, ionizing radiation resulted in a characteristic *in vivo hprt* molecular mutational spectrum characterized by deletions. The spectral change from background is not produced by the cancer itself, as it does not occur in cancer patients pre-RIT. Further analysis showed the average size of *hprt* deletions to be increased by ionizing radiation. Forty-one percent of the gross structural alterations in the radiation associated mutations were total *hprt* deletions, compared to 22% and 15% in normal adults and pre-RIT cancer patients, respectively. Molecular studies using linked X-chromosome DNA anonymous probes and pulsed-field gel electrophoresis have shown that some of the *hprt* deletions are quite large, i.e., up to 2 Mb (71,72). Finally, a correlation was found between the percentage of *in vivo hprt* mutations in an individual showing a gross structural alteration and the cumulative radiation dose received by the individual. In fact, 67% of this variability could be explained by the ionizing radiation exposure. This study indicates that determining the *in vivo hprt* molecular mutational spectrum is valuable in individuals exposed to ionizing radiation, and may be as important as quantitative mutation studies for this particular genotoxic risk recognition.

## Conclusions

Genetic damage manifest as gene mutations can clearly be determined in human somatic cells and the mutagenic effects of exposure to genotoxic agents can be quantified.

The four assays described here provide the ability to detect a complete spectrum of mutagenic effects. The RBC assays have the potential advantage of long memory because of the stem-cell component. The T-lymphocyte assays allow direct molecular characterization of the full range of mutation events and the advantage of providing a temporal relationship between the exposure and the mutation event.

These biomarkers can clearly provide measurement of somatic cell mutations occurring in human and the increases which result from exposure to genotoxic agents. Future studies will determine the correlation of these specific somatic cell effects with human genotoxic diseases and the utility of these assays for risk assessment.

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